SUPPLEMENTARY MATERIAL

Supplementary Methods

Plasmids

The GST-fMDC1 plasmid was generated by inserting a fragment corresponding to amino acid residues 679-778 in pGEX-4T1. The plasmid encoding a GST-p53$^{1-56}$ fusion is a gift from KuDOS Pharmaceuticals. The siRNA-resistant MDC1 expression plasmid was generated by mutating the MDC1 siRNA target sequence in a GFP-MDC1 expression plasmid with 6 silent 'wobble base' mutations in MDC1 (sites of mutations are underlined: 5'-GTC TC<u>G</u> CA<u>A</u> AA<u>G</u> AC<u>G</u> GT<u>C</u> AT<u>C</u>-3'). The YFP-RNF8 and FLAG-RNF8 expression plasmids were purchased from Genecopoeia (www.genecopoeia.com). The mouse RNF8 expression vector was obtained from Open Biosystems. The GST-RNF^{FHA} fusion protein was generated by inserting a fragment encompassing amino acid residues 2-160 of human RNF8 in the EcoRI-XhoI sites of pGEX-4T1. Site-directed mutagenesis to generate the derivatives of the above plasmids was carried out using Quikchange (Invitrogen).

RNA interference

All siRNAs employed in this study (except for those against MDC1 used in Fig. 1) were SMARTpools (from ThermoFisher). The MDC1 siRNA from Fig. 1 (sequence: 5'-GUC UCC CAG AAG ACA GUG ATT-3') was purchased from MWG Biotech AG. esiRNAs were produced exactly as described in (*1*). Oligonucleotide sequences to

produce the esiRNAs were obtained from the RIDDLE database (*2*). All RNAi transfections were performed using Dharmafect 1 (ThermoFisher) or Oligofectamine (Invitrogen) transfection reagents in a forward transfection mode.

Quantitation of 53BP1 foci by high content microscopy

Cell images were captured on the Opera automated spinning-disc confocal microplate imaging microscope (Perkin Elmer). Dichroic filters and two 12-bit Peltier cooled CCD cameras were used to simultaneously detect fluorescently-labeled samples, using an air 20X objective lens. Cell nuclei were detected through a 450 +/- 50 nm bandpass filter upon epifluorescent excitation of DAPI by a UV light source, and 53BP1 foci were detected within a 1 μm confocal slice through a 525 +/- 50 nm filter upon excitation of Alexa 488-conjugated secondary antibody (Molecular Probes) by a 488 nm laser line. Multiple fields of view with two signal channels per field were collected per well of a 384-well plate, corresponding on average to ~40 nuclei per field. Approximately 3000 to 5000 nuclei were analyzed per condition, per imaging experiment.

Image segmentation was performed with the Acapella software bundled with the Opera microscope. For every field of view collected, nuclei and 53BP1 signals were defined and measured using image data captured by the respective CCD cameras dedicated to the UV or 488nm laser light sources. Partial nuclei were discarded from analysis. Attributes for each nucleus describing area, length, width and intensity were measured using DAPI fluorescence image data. Nuclear borders were overlaid onto Alexa 488 fluorescence image data, and 53BP1 signal characterization was restricted to

these nuclear zones. 53BP1 foci were detected using a spot finding algorithm and values describing focus size and intensity were obtained. Data tables describing average and total scores of nuclear and 53BP1 focus attributes were compiled within Acapella and exported for visualization in standard graphing programs. The detailed script for focus detection is available upon request.

Assessment of z-scores for siRNAs

To score siRNAs during the primary screen according to their potency in affecting 53BP1 focus formation, we ranked each quadruplicate transfection according to their z-score, which allowed correcting for plate-to-plate variations. For each RNAi we derived a population mean X and standard deviation σ. To center X around the non-targeting control (*ntc*) we calculated an adjustment factor alpha = median(*X_ntc*)/sd(*X_ntc*) and used the non-targeting control as follows:

$$Z = \frac{(x - \mu) * \alpha}{\sigma}$$

where μ = is the median non-targeting control between wells per plate and σ = the standard deviation of the sample population of X.

Time-lapse microscopy

Three-dimensional image datasets were acquired on a DeltaVision Core imaging system equipped with an Olympus IX71 microscope, a CoolSNAP HQ2 1024x1024 CCD camera (Roper Scientific), and a 60X NA 1.42 using 2x2 binning. Z-stacks were acquired every 5 minutes, computationally deconvolved using the SoftwoRx software package

(v3.6, Applied Precision) and shown as two-dimensional projections.

Antibodies and immunoblotting

Whole-cell extracts, immunoblotting and immunoprecipitation was carried out essentially as described in (*3*). We employed the following antibodies: rabbit anti-RNF8 antibody specific to human RNF8 (*4, 5*), mouse anti-53BP1 (clone 19, BD Biosciences), mouse anti-MDC1 (clone MDC1-50, Sigma-Aldrich), goat anti-MDC1 (AbD, Serotec), sheep anti-MDC1 (*6*), mouse anti-γ-H2AX (clone JBW301, Upstate), rabbit anti-γH2AX (Cell Signaling Technologies), rabbit anti-FANCD2 (Novus Biologicals), mouse anti-BRCA1 (clone MS110, Calbiochem; clone D9, Santa Cruz), rabbit anti-RAP80 (Bethyl Laboratories), mouse anti-histone H3 phosphoserine 10 (clone 6G3, Cell Signaling Technologies), mouse anti-poly-ubiquitin (clone FK2, Stressgen). 1), mouse anti-GST (clone B4, Santa Cruz), mouse anti-ATM (clone mat3, a gift from Y. Shiloh), rabbit anti-Nbs1/p95 (Novus Biologicals), rabbit anti-53BP1 (Novus Biologicals), rabbit anti-Chk2 pT68 (Calbiochem), goat anti-Chk2 (Santa Cruz), rabbit anti-SMC1 and anti-SMC1 pS966 (Bethyl). The MDC1 pT719 antiserum was raised and affinity-purified against a phospho-peptide encoding MDC1 amino acid residues 713-725 by Abcam Plc (Cambridge, UK) and an additional rabbit anti-RNF8 antibody was raised against recombinant RNF8 (amino acid residues 343-485).

ATM kinase assays

1 μg of rabbit anti-ATM antibody (a gift of KuDOS Pharmaceuticals) or pre-immune serum was added to 500 μg of HeLa nuclear extracts (www.cilbiotech.be) in an equal volume of IP buffer (25 mM HEPES pH 7.4, 2 mM $MgCl_2$, 25 0mM KCl, 10% glycerol, 0.5 mM EGTA, 0.1 mM $NaVO_4$, 0.1% M NP-40) and incubated at 4°C for 2 hrs. Immune complexes were subsequently isolated with protein G sepharose (Sigma-Aldrich) for 1 hr before washing with kinase buffer (10 mM HEPES pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1mM DTT, 1 X protease inhibitors (EDTA-free Complete tablets, Roche)). Kinase reactions were assembled in a total volume of 25 μL in kinase buffer with 500 ng of the indicated GST fusion protein, ATP at 40 μM concentration and spiked with 1 μL $\gamma[^{32}P]$-ATP (Amersham). Reactions were incubated for 30 min at 30°C and then stopped by addition of SDS-PAGE sample buffer. Reactions were resolved by SDS-PAGE and transferred to PVDF.

Recombinant protein production

Expression of recombinant protein in *E. coli* strain BL21(DE3) pLysS was induced at 37°C when bacterial cultures reached OD_{600} of 0.8 using 1 mM 1-β-D-thiogalactopyranoside. Fusion proteins were usually purified on glutathione-Sepharose 4B according to the batch method described in the GST manual (Pharmacia).

Surface Plasmon resonance

For surface plasmon resonance, biotinylated peptides were immobilized on a streptavidin-coated chip (SA5) using a BIAcore 3000 (GE Healthcare). Biotinylated

peptide (0.1 mM) was manually injected at 10 μL/min to an equivalent of 400 resonance units (RU). The GST-FHA fusions were injected at a flow rate of 10 μL/min. To regenerate the surface after each sample, 10 mL of 0.5% SDS in 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.3% NP-40, 1 mM DTT, 1 mM PMSF were injected. This proved sufficient to dissociate completely the FHA-peptide complex without affecting the streptavidin-peptide complex. The temperature was maintained at 4°C for the duration of the experiment.

Immunofluorescence microscopy

HeLa or U2OS cells were grown on glass coverslips. Cells were washed with PBS twice before fixation. For γH2AX, MDC1, 53BP1, phospho-ATM and poly-ubiquitin immunodetection, cells were fixed with methanol at -20^{o}C for over 1 hr and then permeabilized with acetone at -20^{o}C. For FANCD2 immunodetection, cells were fixed with 1-2% paraformaldehyde, 0.2% Triton X-100 in PBS for 30 min at room temperature. After fixation, cells were washed with PBS four times and then blocked with 5% normal goat serum, 0.1% Triton X-100 in PBS for 1 hr. Cells were stained with a γ-H2AX mouse monoclonal antibody (Upstate) at a dilution of 1:5000. Antibodies were diluted in 5% ADB (5% normal goat serum, 0.1% Triton X-100 in PBS). Cells were washed with 5% ADB and then stained with Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 555 goat anti-rabbit IgG, Alexa Fluor 647 goat anti-rabbit IgGor Alexa fluor 555 donkey anti-sheep IgG antibodies (Molecular Probes) for 1 hr at room temperature. Secondary antibodies were diluted in 5% ADB. For RAP80 and BRCA1 immunodetection, cells

were treated with nuclear extraction buffer (20 mM HEPES, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride, 1X Protease Inhibitor Cocktail [Complete, EDTA-free; Roche], 0.5% IGEPAL CA-630; pH 7.5) for 20 min on ice, followed by fixation with 2% paraformaldehyde in PBS, blocked with 10% ADB (10% normal goat serum, 0.05% Triton X-100 in PBS), then immunostained with anti-RAP80 and anti-γH2AX antibodies diluted in 10% ADB, overnight at 4°C. Cells were washed with PBS, counterstained with goat anti-rabbit IgG Alexa Fluor 488 and goat anti-mouse IgG Alexa Fluor 555 (Molecular Probes) secondary antibodies diluted 1:1000 in 10% ADB, for 1 hr at room temperature. DNA was counterstained with DAPI (0.2 μg/ml) in PBS, washed with PBS and mounted with Prolong Gold mounting agent (Invitrogen). Confocal images were taken using Leica DMIRE2 microscope or BioRad Radiance 2100 (on a Nikon Eclipse E800 - upright) microscopes, equipped with 63X and 40X oil lenses. Images were acquired with Volocity Software v. 4.1, or Lasersharp 2000 (Zeiss).

Peptide pull-downs

Peptide pull-downs were carried out by mixing 1 mg of HeLa nuclear extracts and 30 μL streptavidin Dynabeads (Dynal) saturated with the following biotinylated peptides.

MDC1 T719: Biotin-SGS-AVQSMEDEPTQAFML

MDC1 pT719: Biotin-SGS-AVQSMEDEPpTQAFML (pT denotes phosphothreonine)

MDC1 T752: Biotin-SGS-LDEPWEVLATQPFCL

MDC1 pT752: Biotin-SGS-LDEPWEVLApTQPFCL (pT denotes phosphothreonine)

All incubations and washes were carried out in the following buffer: 150 mM NaCl, 3 mM KCl, 25 mM Tris pH 8.0, 10% glycerol, 1 mM DTT, 1 X protease inhibitors (complete tablets, mini EDTA-free, Roche).

Clonogenic survival assays.

HeLa cells in 100 mm dishes were transfected twice with control, ATM and RNF8 siRNAs at 24 hour time intervals. 24 hrs after the second transfection, cells were split and transferred into 60 mm dishes for irradiation 12 hrs later. The clonogenic assay was set up exactly as in (*7*). Cells were irradiated using a Faxitron X-ray cabinet at 3.125 Gy/min dose rate, at various doses, and immediately transferred into 6-well dishes. 14 days after irradiation cells were washed with PBS, fixed and stained with a mix of 20% methanol/0.5% Crystal Violet. Colonies containing 50 or more cells were counted using a stereo-dissecting microscope.

G2/M checkpoint analysis

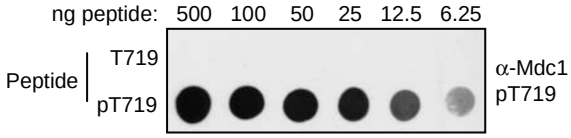
U2OS cells were exposed to 2 Gy IR 48 hrs after siRNA transfection. Cells were harvested 1 hr after IR. Cells were washed with PBS twice and then fixed with 1% paraformaldehyde at 37^{o}C for 10 min. After cooling on ice for 1 min, cells were permeabilized with 90% methanol and stored at -20^{o}C overnight. Fixed cells were washed with PBS twice and blocked with FACS incubation buffer (0.5% BSA in PBS) for 10 min. Cells were then stained with anti-phospho-Histone H3 (Ser10) antibody at 1:25 dilution in FACS incubation buffer for 1 hr at room temperature. All washes were

carried out in FACS incubation buffer. For secondary antibody staining, FITC-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch), diluted 1:100 was used and cells were incubated for 1 hr at room temperature. After secondary antibody staining, cells were washed and then incubated with 5 μg/mL propidium iodide (Sigma-Aldrich) and 250 μg/mL RNase I in PBS. For ATM inhibitor assay, cells were treated with 10 μM of KU-55933 (kind gift of Graeme Smith, KuDOS Pharmaceuticals) 20 min prior to irradiation. More than 10,000 cells were analyzed by flow cytometry (Becton Dickinson). Data were analyzed by Cell Quest Pro (Becton Dickinson).


TQXF 1
TQXF 2
TQXF 3
TQXF 4
hMdc1 650 VDTDTLGESTQPQREGAQVPTGREREQHVGGTKDSEDNYGD-SEDLDLQATQCFLE--NQ
ptMdc1 650 VDTDTLGESTQPQREGAQVPTGREREQHVGGTKDSEDNYGD-SEDLDLQATQCFLE--NQ
ssNFBD1 641 VDTGAPGEATQTQREGTQALTERERELNVDRTIDSGDNRDD-SEDLDLQATQCFVEGENQ
cfMdc1 644 VDTGTSGETIQPQKEGAQTPTERKREPHVDRTKNCGDNHGD-SEDLDLQATQCFVERENQ
mmMdc1 605 IHTDTSGDPTLPQREGAQTPTGREREAHVGRTKSAKECCDAEPEDLCLPATQCFVEGESQ
hMdc1 707 GLEAVQSMEDEPTQAFMLTPPQELGPSHCSFQTTG--TLDEPWEVLATQPFCLRE-SEDS
ptMdc1 707 GLEAVQSMEDEPTQAFMLTPPQELGPSHCSFQTTG--TLDEPWEVLATQPFCLRE-SEDS
ssNFBD1 700 SPEAVPSMEDEPTQAFPSTLPQEPGPSCCSFQASG--TLDEPWELLATQPFCPRE-SEAS
cfMdc1 703 SPEAVQSMEDEATQAFLATLPQESGPSYYSFQASG--ALDEPWEVLATQPFCPRE-SEAS
mmMdc1 665 HPEAVQSLENEPTQLFPCTLPQEPGPSHLSLQTPGADTLDVPWEVLATQPFCLREQSETS
hMdc1 764 ETQPFDTHLEAYGPCLSPPRAIPGDQHP--ESPVHTEPMGIQGRGRQTVDKVMGIPKETA
ptMdc1 764 FTQPFDTHLEAYGPCLSPPRAIPGDQHP--ESPVHTEPMGIQGRGRQTVDKVMGIPKETA
ssNFBD1 757 EIQPVDTHLEARGLCPSPPRAALPEQHP--ESP---EPLGSQGGGRQTVEKATGTPRETA
cfMdc1 760 EPQPIAAHIDAHGSCPSTPRTAPQAQHP--ESPVHAEPLGIQGRGMQTVEKDM-------
mmMdc1 725 ELH-----EAHGSQPSLPREPPGHQHLVHTSPVHTELLRIEGREIQTVEKAMGIPKEMA

Figure S1

A


ng peptide: 500 100 50 25 12.5 6.25
Peptide
T719
pT719
α-Mdc1
pT719

B

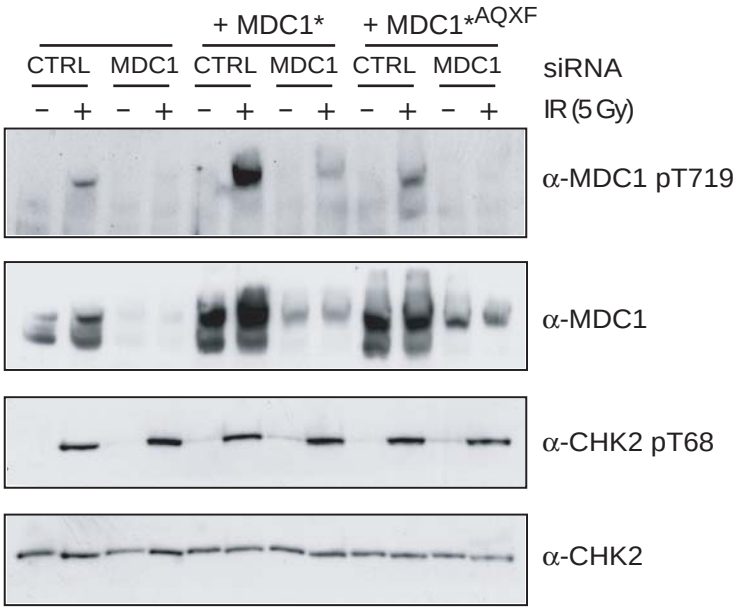

+ MDC1*
+ MDC1*AQXF
CTRL MDC1 CTRL MDC1 CTRL MDC1
siRNA
− + − + − + − + − + − +
IR (5 Gy)
α-MDC1 pT719
α-MDC1
α-CHK2 pT68
α-CHK2

Figure S2

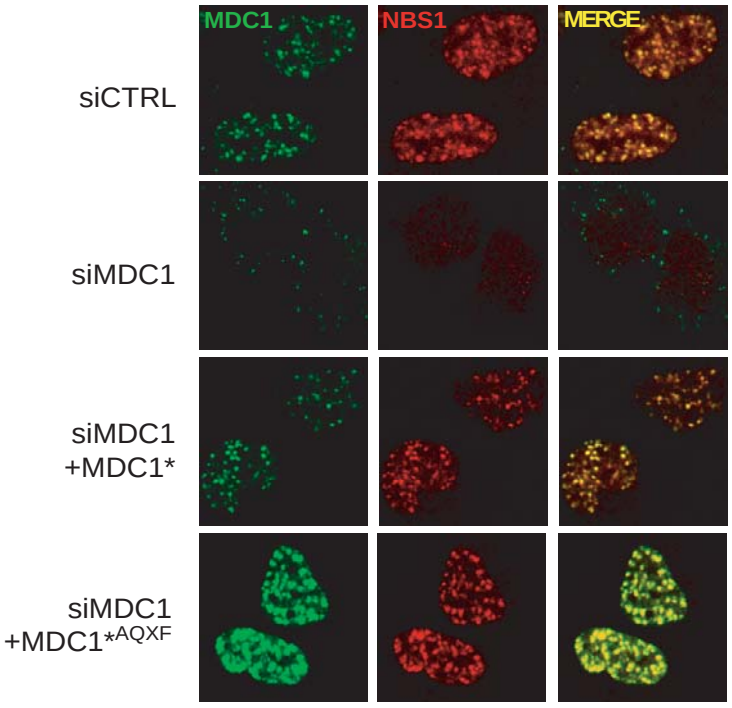
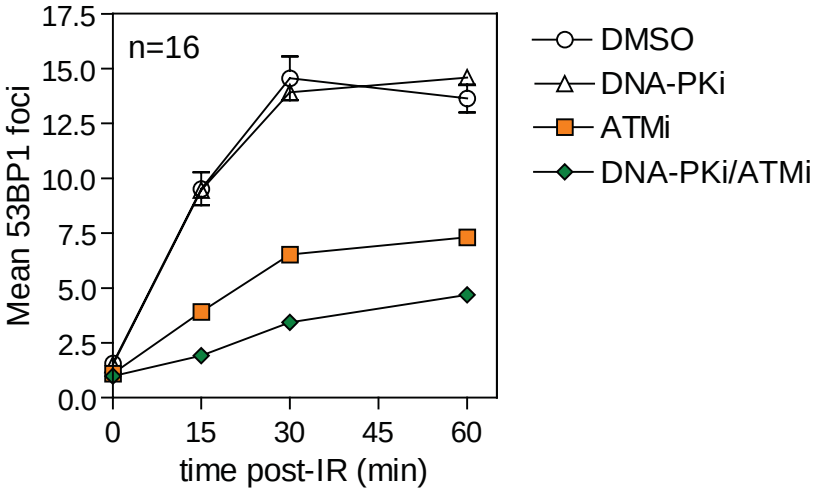


Figure S3

A


n=16
Mean 53BP1 foci
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0
15
30
45
60
time post-IR (min)
DMSO
DNA-PKi
ATMi
DNA-PKi/ATMi

B

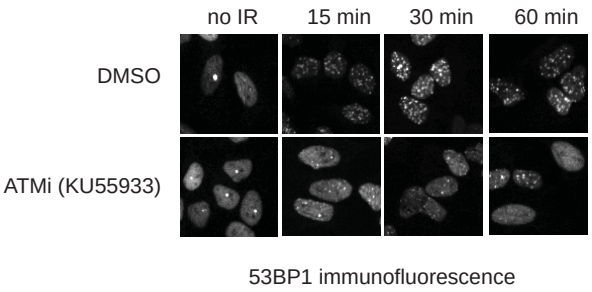

no IR
15 min
30 min
60 min
DMSO
ATMi (KU55933)
53BP1 immunofluorescence

Figure S4

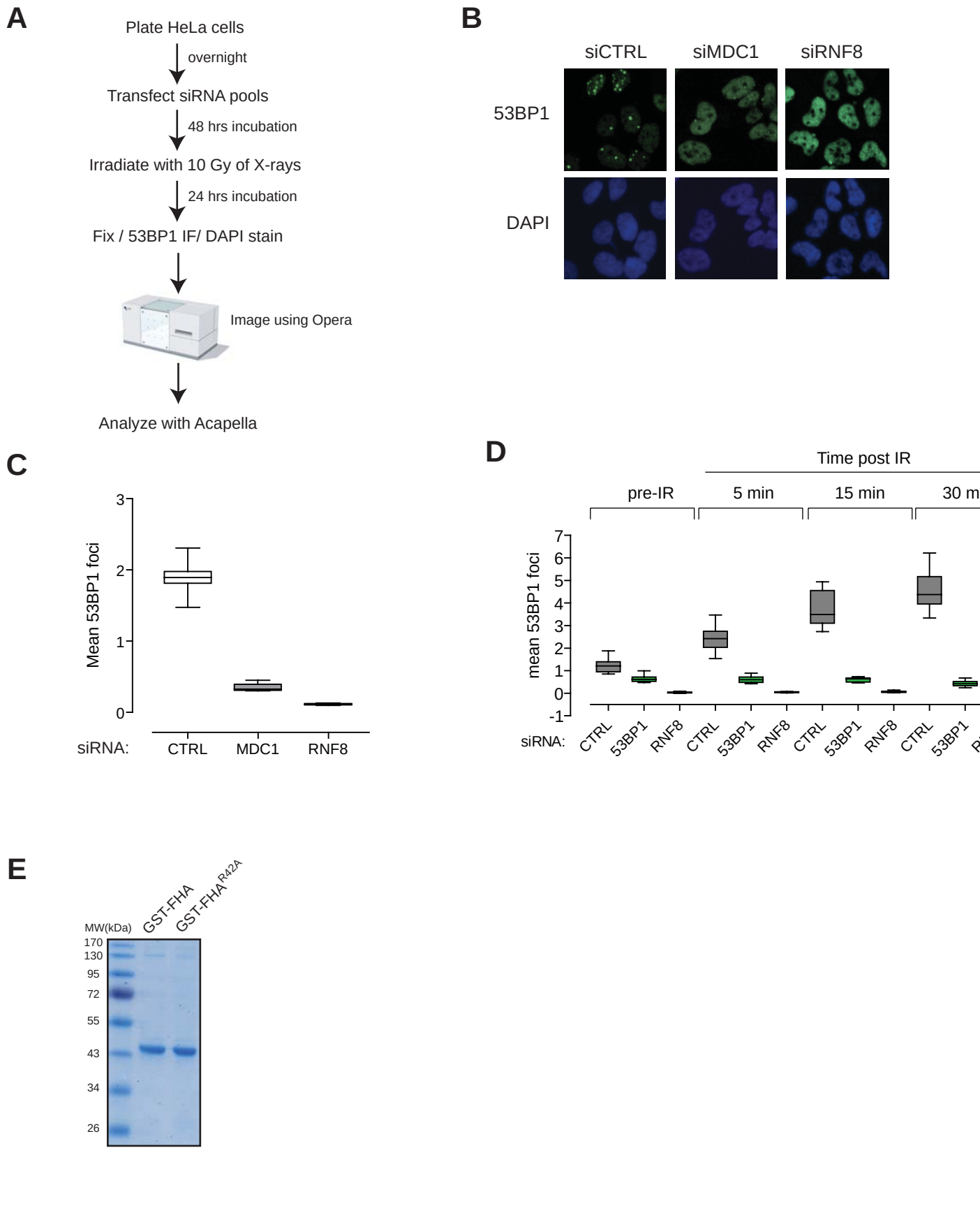

A
Plate HeLa cells
overnight
Transfect siRNA pools
48 hrs incubation
Irradiate with 10 Gy of X-rays
24 hrs incubation
Fix / 53BP1 IF/ DAPI stain
Image using Opera
Analyze with Acapella
B
siCTRL
siMDC1
siRNF8
53BP1
DAPI
C
Mean 53BP1 foci
siRNA:
CTRL
MDC1
RNF8
D
Time post IR
pre-IR
5 min
15 min
30 min
mean 53BP1 foci
siRNA:
CTRL
53BP1
RNF8
E
MW(kDa)
GST-FHA
GST-FHA R42A
170
130
95
72
55
43
34
26

Figure S5

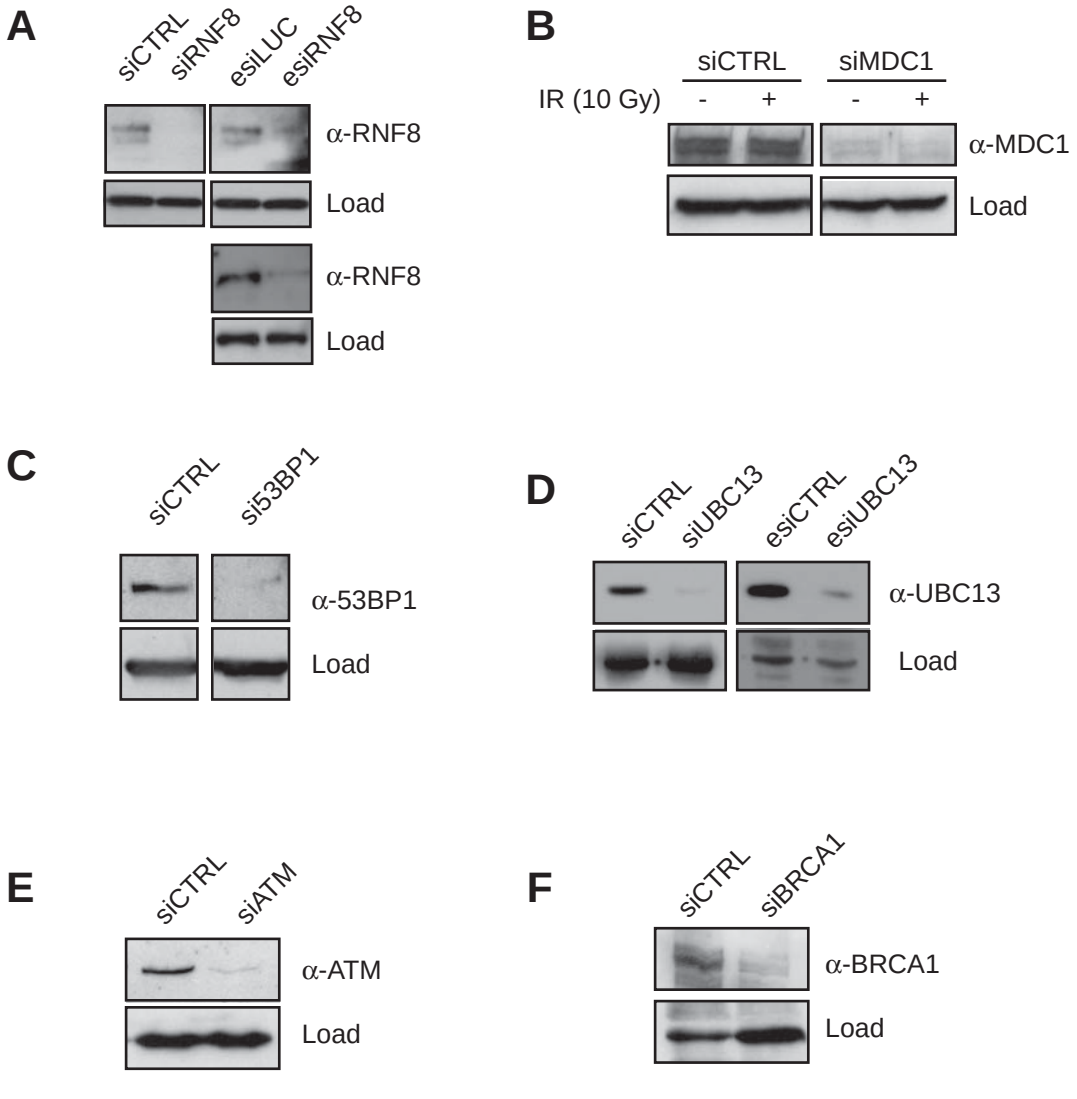
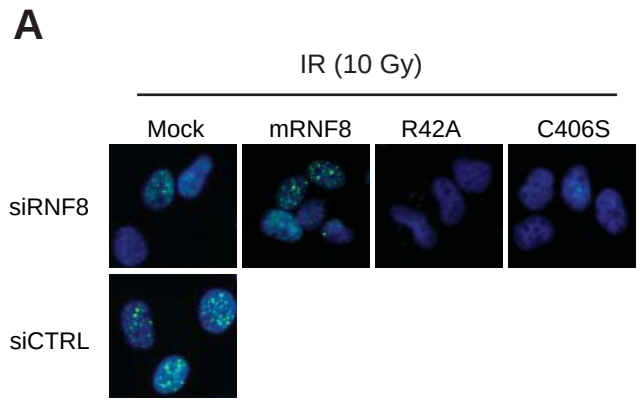

A
siCTRL
siRNF8
esiLUC
esiRNF8
α-RNF8
Load
α-RNF8
Load
B
siCTRL
siMDC1
IR (10 Gy)
-
+
-
+
α-MDC1
Load
C
siCTRL
si53BP1
α-53BP1
Load
D
siCTRL
siUBC13
esiCTRL
esiUBC13
α-UBC13
Load
E
siCTRL
siATM
α-ATM
Load
F
siCTRL
siBRCA1
α-BRCA1
Load

Figure S6


A
IR (10 Gy)
Mock
mRNF8
R42A
C406S
siRNF8
siCTRL

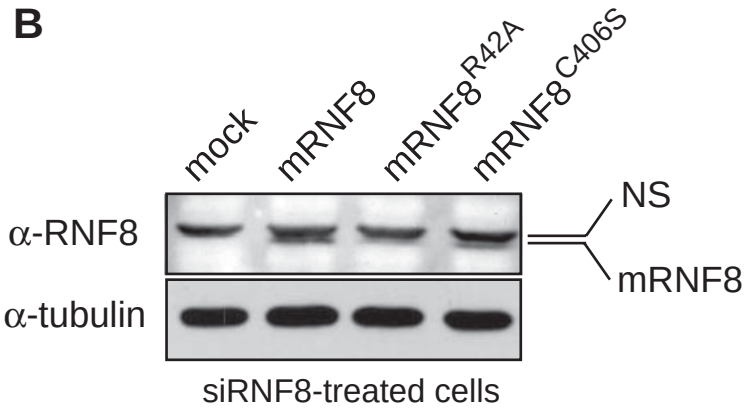
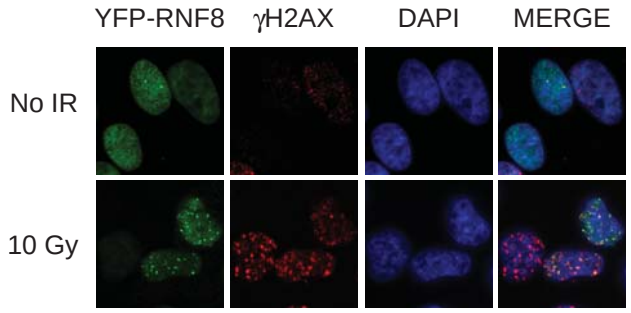
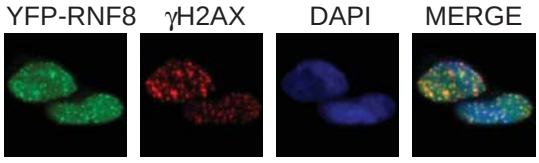

B
mock
mRNF8
mRNF8R42A
mRNF8C406S
α-RNF8
α-tubulin
NS
mRNF8
siRNF8-treated cells

Figure S7

A



B



15 min post-phleomycin treatment

Figure S8

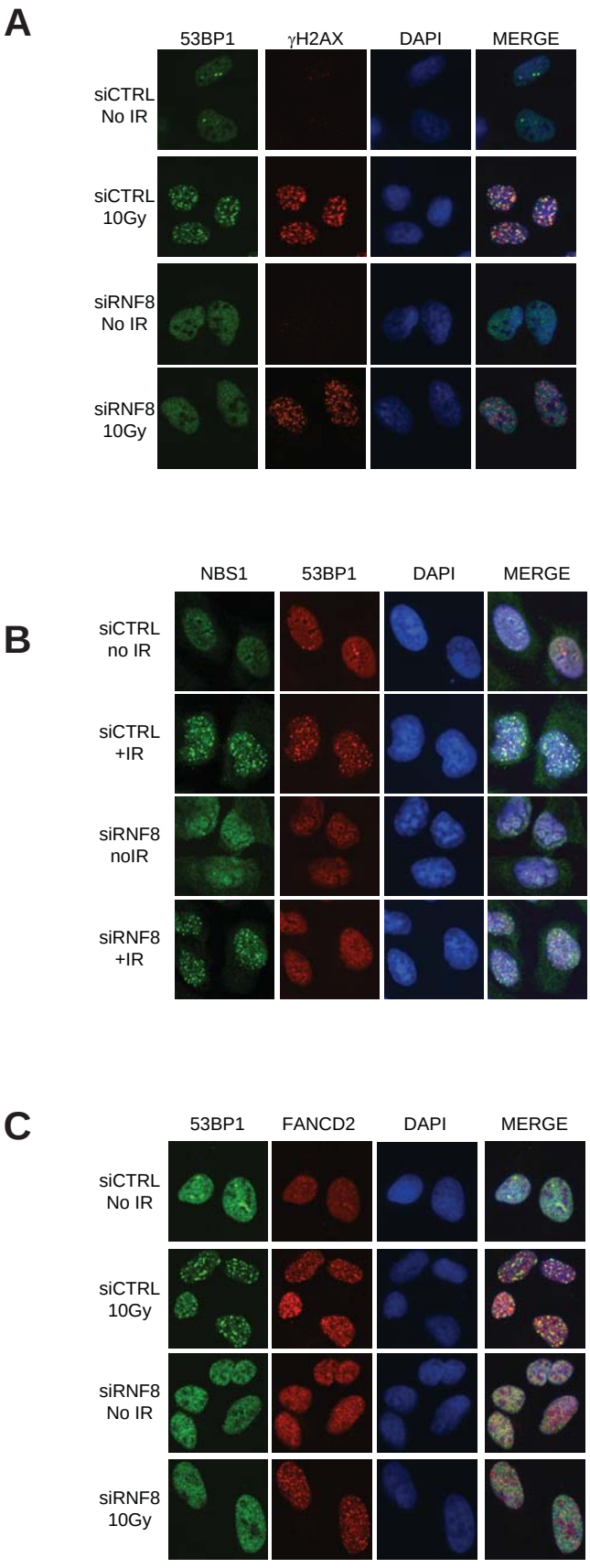

A
53BP1
γH2AX
DAPI
MERGE
siCTRL No IR
siCTRL 10Gy
siRNF8 No IR
siRNF8 10Gy
B
NBS1
53BP1
DAPI
MERGE
siCTRL no IR
siCTRL +IR
siRNF8 noIR
siRNF8 +IR
C
53BP1
FANCD2
DAPI
MERGE
siCTRL No IR
siCTRL 10Gy
siRNF8 No IR
siRNF8 10Gy

Figure S9

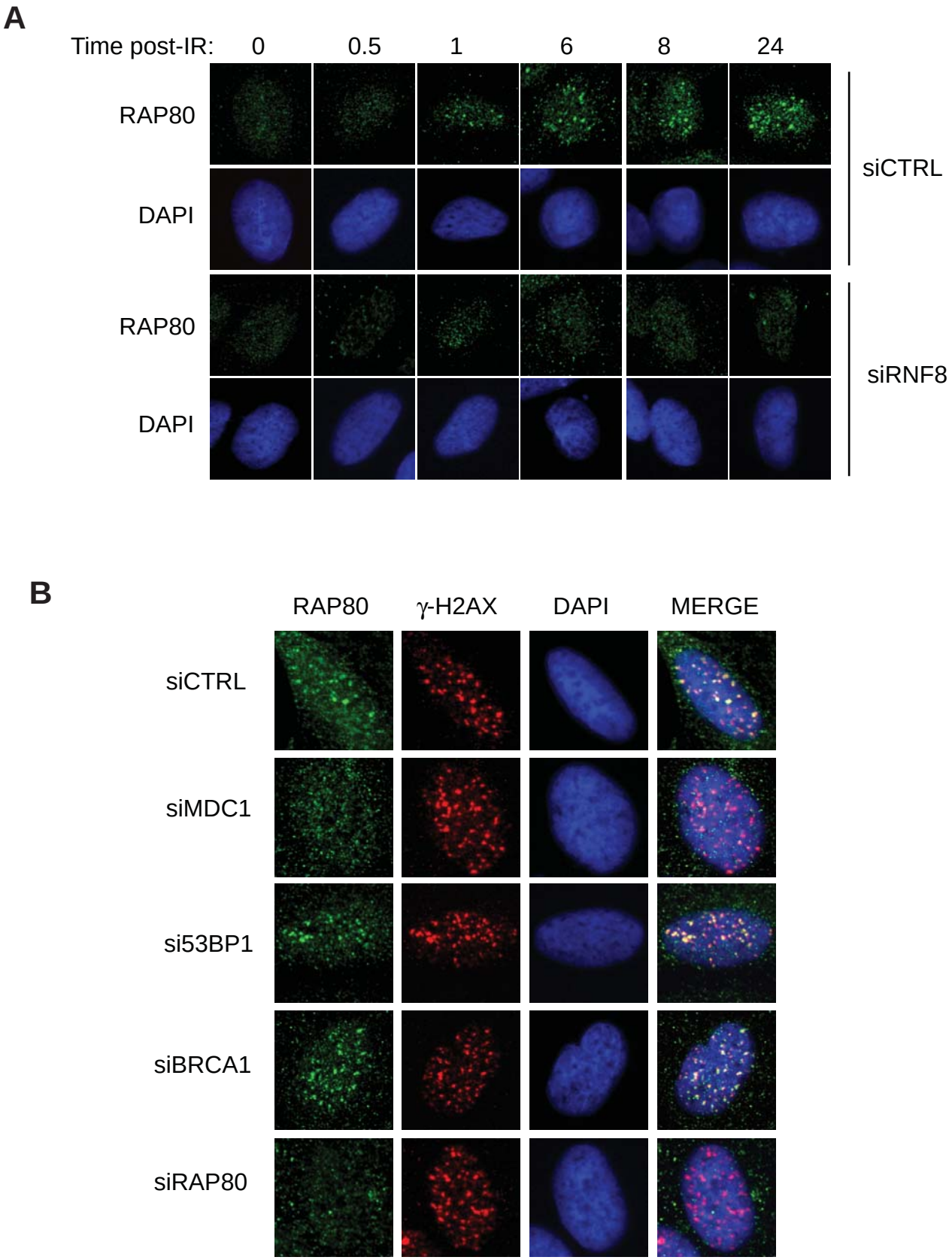

A
Time post-IR: 0 0.5 1 6 8 24
RAP80
DAPI
siCTRL
RAP80
DAPI
siRNF8
B
RAP80
γ-H2AX
DAPI
MERGE
siCTRL
siMDC1
si53BP1
siBRCA1
siRAP80

Figure S10

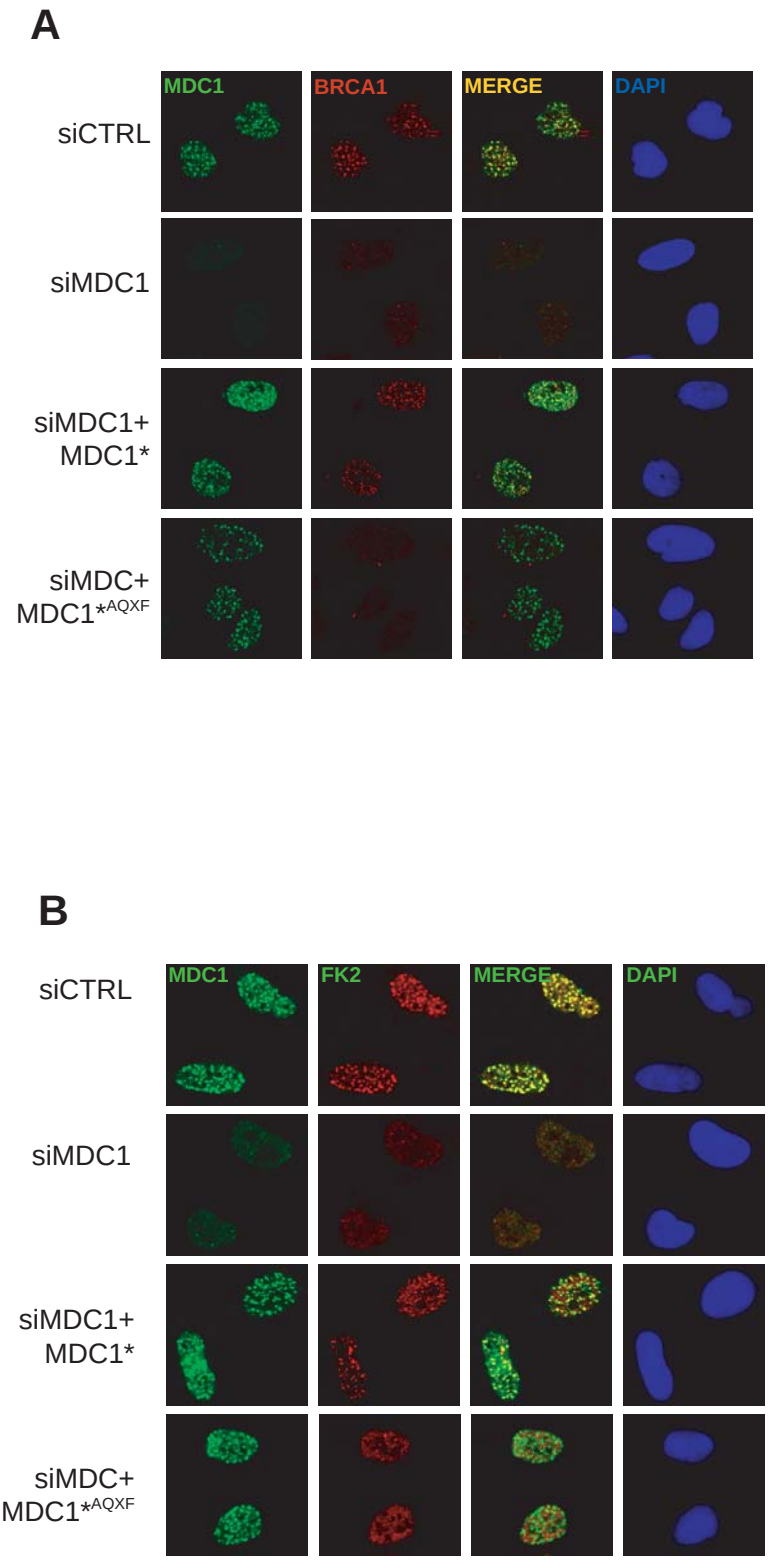

A
MDC1
BRCA1
MERGE
DAPI
siCTRL
siMDC1
siMDC1+
MDC1*
siMDC+
MDC1*AQXF
B
MDC1
FK2
MERGE
DAPI
siCTRL
siMDC1
siMDC1+
MDC1*
siMDC+
MDC1*AQXF

Figure S11

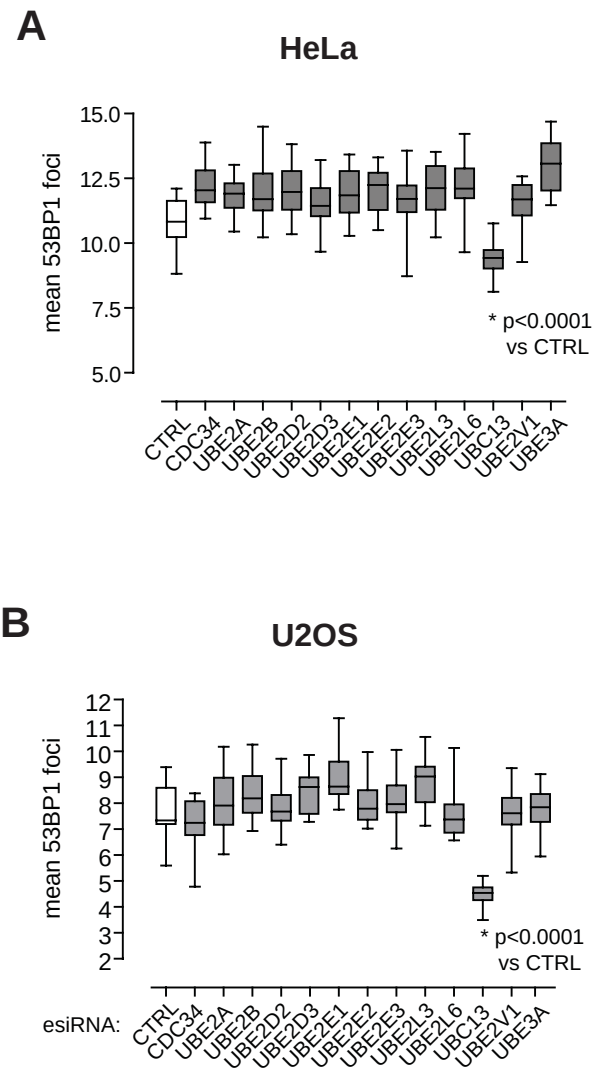

A
HeLa
mean 53BP1 foci
15.0
12.5
10.0
7.5
5.0
* p<0.0001
vs CTRL
CTRL
CDC34
UBE2A
UBE2B
UBE2D2
UBE2D3
UBE2E1
UBE2E2
UBE2E3
UBE2L3
UBE2L6
UBC13
UBE2V1
UBE3A
B
U2OS
mean 53BP1 foci
12
11
10
9
8
7
6
5
4
3
2
* p<0.0001
vs CTRL
esiRNA:
CTRL
CDC34
UBE2A
UBE2B
UBE2D2
UBE2D3
UBE2E1
UBE2E2
UBE2E3
UBE2L3
UBE2L6
UBC13
UBE2V1
UBE3A

Figure S12

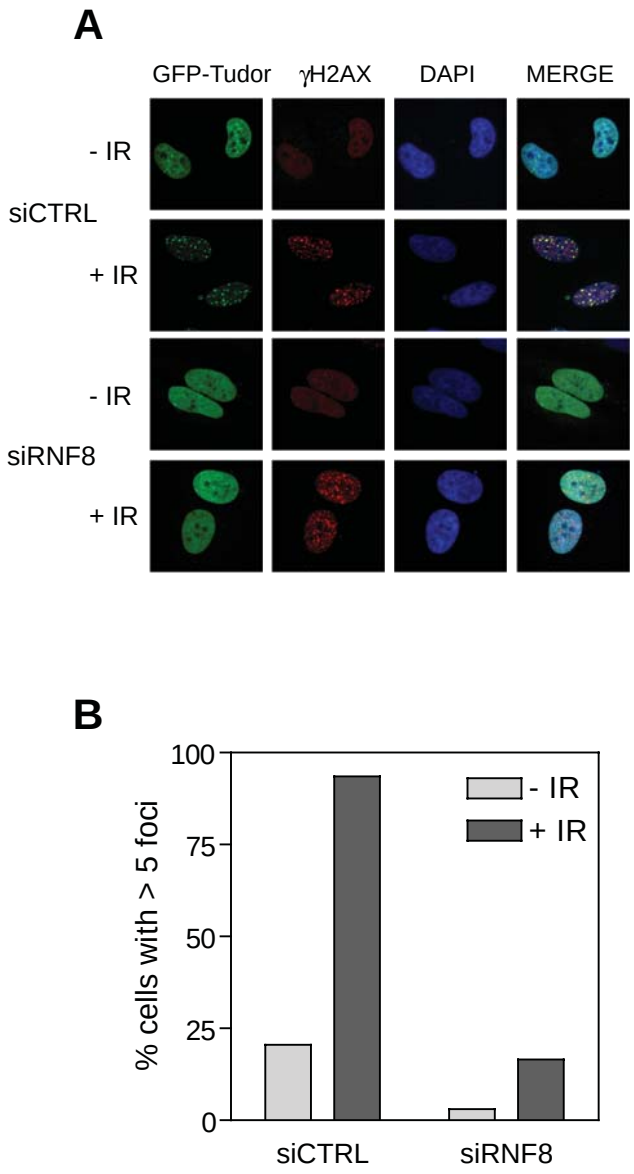

A
GFP-Tudor
γH2AX
DAPI
MERGE
- IR
siCTRL
+ IR
- IR
siRNF8
+ IR
B
% cells with > 5 foci
100
75
50
25
0
- IR
+ IR
siCTRL
siRNF8

Figure S13

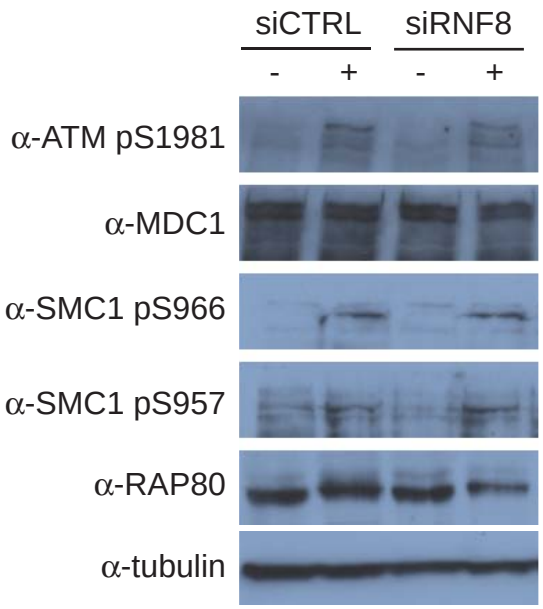

siCTRL
siRNF8
-
+
-
+
α-ATM pS1981
α-MDC1
α-SMC1 pS966
α-SMC1 pS957
α-RAP80
α-tubulin

Figure S14

Supplementary Figure legends

Fig. S1. The MDC1 N-terminus contains a conserved cluster of motifs sharing the TQXF consensus. Shown is a multiple sequence alignment of the MDC1 TQXF region in human (h), chimp (pt), pig (ss), dog (cf), and mouse (mm) MDC1 protein sequences. The black shaded squares indicate identical residues, and grey shaded squares indicate similar residues. Green boxes highlight positions of the TQXF motifs 1-4.

Fig. S2. (**A**) Specificity of the MDC1-pT719 antibody. Indicated amounts of phosphorylated and non-phosphorylated MDC1 T719 peptide were spotted onto nitrocellulose. The filter was subsequently probed using the anti-MDC1 pT719 antibody. (**B**) Expression vectors encoding siRNA-resistant GFP-MDC1 (MDC1*) or GFP-MDC1^{AQXF} (MDC1*^{AQXF}) were stably transfected into U2OS cells, and clones were selected for endogenous levels of MDC1 expression. Cells were then transfected with an siRNA against MDC1, or luciferase (CTRL). 72 hrs after siRNA transfection, cells were irradiated with 5 Gy, harvested ten minutes post-IR and processed for MDC1 pT719, MDC1, CHK2 and CHK2 pT68 immunoblotting.

Fig. S3. The TQXF cluster is not required for NBS1 IRIF. U2OS cells stably transfected with vectors encoding siRNA-resistant GFP-MDC1 (MDC1*) or GFP-MDC1^{AQXF}

(MDC1*^{AQXF}) were transfected with siRNAs against MDC1 (siMDC1) or luciferase (siCTRL) and stained for NBS1 and MDC1 immunofluorescence post-irradiation (5Gy).

Fig. S4. ATM inhibition curtails 53BP1 recruitment into IRIF. (**A**) Quantitation of 53BP1 IRIF formation prior following 10 Gy IR in HeLa cells incubated with DMSO, ATM inhibitor (ATMi; KU55933), DNA-PK inhibitor (DNA-PKi; NU7026) or a 1:1 combination of the DNA-PK and ATM inhibitors. Cells were fixed at the indicated time points following irradiation and 53BP1 foci were visualized and quantitated, as described in materials and methods. At least 500 nuclei per well were imaged. N=16 +/-SD. (**B**) Representative Opera microscope screen captures of the experiment quantitated in (A) for the DMSO and ATM inhibitor conditions.

Fig. S5. Identification of RNF8 as a gene required for 53BP1 IRIF formation in a siRNA screen. (**A**) Schematics of the ongoing siRNA screen which led to the identification of RNF8. (**B**) Opera microscope screen captures from the siRNA screen where HeLa cells were transfected with non-targeting (siCTRL), RNF8, or MDC1 siRNAs as indicated. The cells were fixed 24 hrs post 10 Gy irradiation. (**C**) Quantitation of 53BP1 foci from the primary screening data. "Mean 53BP1 foci" describes the output from a custom-built 53BP1 focus detection script and refers to the average number of 53BP1 foci detected per cell in a confocal 1 μm z-section. At least 500 nuclei per well were imaged. Data from 16 transfected wells per condition is displayed using box-and-whisker plots. (**D**) RNF8 mediates 53BP1 recruitment rather than maintenance of IRIF. Quantitation of 53BP1

IRIF formation in the indicated siRNA-transfected HeLa cells prior (pre-IR) or following 10 Gy irradiation at the indicated time points. IRIF data from 16 wells per condition and analyzed as described in (D). The data is represented by box-and-whisker plots. (**E**) Coomassie staining of the purified GST-RNF8^{FHA} and GST-RNF8$^{FHA-R42A}$ used for the surface plasmon resonance experiments shown in Fig. 3A.

Fig. S6. Validation of RNAi reagents by immunoblotting. (**A**) Whole-cell extracts (1-3 mg) from cells treated with the indicated RNAi reagents were incubated with immoblilized pT719 peptides and immunoblotted with an anti-RNF8 antibody (*4*). Two independent validations of esiRNF8 are shown. Actin was used as loading control. (**B-F**) HeLa or U2OS whole cell lysates were immunoblotted with the indicated antibodies. Actin (D) or tubulin (B, C, E, F) were used as a loading control.

Fig. S7. (**A**) Representative Opera microscope screen captures from HeLa cells transfected with non-targeting (siCTRL) or RNF8 siRNAs as indicated. Cells were then replated and transfected with siRNF8-resistant murine RNF8 (WT) or its FHA (R42A) or RING finger (C406S) mutated versions. The cells were fixed 24 hrs post 10 Gy irradiation. Quantitation of this data is shown in Fig. 2F. (**B**). Immunoblotting of whole cell extracts of HeLa cells depleted in human RNF8 by siRNA transfection and either mock-transfected or transfected with the indicated murine RNF8 constructs. The R42A mRNF8 mutant is under-expressed relative to wild-type mRNF8 but still overexpressed relative to human RNF8. NS indicates a band that cross-reacts with the RNF8 antiserum.

Fig. S8. YFP-RNF8 forms foci that colocalize with γH2AX. (**A**) HeLa cells were fixed prior (no IR) or following 10 Gy IR and processed for γH2AX immunofluorescence. YFP fluorescence was visualized directly. (**B**) Cells from a 293T clone stably expressing YFP-RNF8 were incubated with phleomycin (1.5 mg/ml) for 15 min before fixation and processed as described in (A).

Fig. S9. RNF8 is not required for γH2AX, NBS1 or FANCD2 IRIF formation (**A-C**) HeLa (A-B) or U2OS (C) cells were mock-treated or transfected with either control (siCTRL) or RNF8 (siRNF8) siRNAs. Cells were then irradiated with 10 Gy and fixed 1 hr post-irradiation and processed for immunofluorescence with antibodies against the indicated proteins.

Fig. S10. (**A**) RAP80 IRIF formation is impaired by RNF8 depletion at all time point tested. U2OS cells transfected with siRNAs against RNF8 (siRNF8) or a non-targeting control (siCTRL) and were fixed at the indicated time points after 10 Gy IR and processed for RAP80 and γH2AX immunofluorescence. (**B**) MDC1, but not 53BP1, is required for RAP80 IRIF. HeLa cells transfected either with a non-targeting siRNA (siCTRL) or with siRNAs targeting MDC1, 53BP1, BRCA1 or RAP80 as indicated were fixed 1 hr following 10 Gy irradiation and processed for RAP80 or γ-H2AX immunofluorescence.

Fig. S11. The TQXF cluster is required for BRCA1 and poly-ubiquitin IRIF. U2OS cells stably transfected with vectors encoding siRNA-resistant GFP-MDC1 (MDC1*) or GFP-MDC1^{AQXF} (MDC1*^{AQXF}) were transfected with siRNAs against MDC1 (siMDC1) or luciferase (siCTRL) and stained for BRCA1 and poly-ubiquitin (FK2) immunofluorescence post-irradiation (5Gy).

Fig. S12. Quantitation of 53BP1 IRIF formation in cells transfected with esiRNAs against the indicated E2 conjugating enzymes in irradiated (10 Gy) (A) HeLa and (B) U20S cells. Cells were fixed 1 hr post irradiation and processed for imaging on the Opera microscope as described in Fig. 2A. The box-plot graph is represents the data acquired on 16 wells. An unpaired t-test was calculated to assess the significance of the difference between the control esiRNA and the esiRNA against UBC13.

Fig. S13. RNF8 mediates the recruitment of the 53BP1 Tudor domain to sites of DNA damage. (**A**) HeLa cells were first transfected with siRNAs against RNF8 (siRNF8) or a non-targeting siRNA (siCTRL) followed by transfection with a construct encompassing the GFP-Tudor domain of 53BP1 (*8*) and were finally mock-treated or irradiated (10 Gy). Cells were fixed 1 hr post-IR and then processed for GFP fluorescence and anti-gH2AX immunofluorescence microscopy. (**B**). Quantitation of the experiment in (A). Over 150 cells were counted for each condition.

Fig. S14. RNF8 acts downstream of ATM. Whole-cell extracts from U2OS cells transfected with either control (siCTRL) or RNF8 (siRNF8) siRNAs were mock or irradiated with 10 Gy and analyzed 1 hr post-IR with the indicated antibodies.

Supplementary references

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